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GRANT NUMBER DAMD17-96-1-6032

TITLE: Factors Modulating Estrogen Receptor Activity

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REPORT DATE: July 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1998	Annual (1 Jul 97 - 30 Jun 98)

4. TITLE AND SUBTITLE Factors Modulating Estrogen Receptor Activity

5. FUNDING NUMBERS
DAMD17-96-1-6032

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
New York University Medical Center
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

19981229 094

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

The mechanism of signal transduction by the estrogen receptor (ER), an estrogen-dependent transcription factor that plays a role in the initiation and progression of breast cancer, is complex and not fully understood. In addition to ER, a number of accessory proteins are apparently required to efficiently transduce the steroid hormone signal. In the absence of estradiol, ER, like other steroid receptors, is complexed with Hsp90 and other molecular chaperone components, including an immunophilin, and p23. This Hsp90-based chaperone complex is thought to repress ER's transcriptional regulatory activities while maintaining the receptor in a conformation that is competent for high affinity steroid binding. However, a role for p23 in ER signal transduction has not been demonstrated. Using a mutant ER (G400V) with decreased hormone binding capacity as a substrate in a dosage suppression screen in yeast (S. cerevisiae), we identified the Hsp90-associated co-chaperone p23 as a positive regulator of ER function in yeast and breast cancer cells. Together, our results strongly suggest that p23 plays an important regulatory role in ER signal transduction and as such, may be exploited in the development of new therapies for estrogen-dependent malignancies, such as breast cancer.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer estrogen receptor molecular chaperones p23	40
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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5. INTRODUCTION

Estrogen is a steroid hormone responsible for the proper function of a variety of mammalian physiological processes. In addition to its central role in reproduction(6, 32), estrogen also affects the cardiovascular(19), skeletal(43), immune(9), and nervous systems(51) and plays a role in the initiation and progression of breast cancer(52).

The estrogen signal is mediated by the estrogen receptor (ER), a ligand-inducible transcription factor. In the absence of estradiol, ER is found predominantly in the nucleus(31, 50), as part of a multiprotein complex consisting of a dimer of Hsp90(6), a p23 monomer(38), and one of several immunophilins, including Cyp-40(45) and FKBP52(44). It has been proposed that this Hsp90-based chaperone complex inactivates ER's transcriptional regulatory capabilities and maintains ER in a conformation competent for steroid binding(42). Upon binding estradiol, ER dissociates from this complex, dimerizes and recognizes specific DNA sequences(34), termed estrogen-response elements (EREs), within the promoters of estrogen-responsive genes. Once bound to an ERE, ER is believed to modulate transcription of the linked gene through direct or indirect interactions with general transcriptional factors(15, 24).

Although many aspects of ER signaling remain to be understood, it appears that the proteins essential to ER function are conserved among eukaryotes to such an extent that introduction of ER into *S. cerevisiae*, which lack endogenous ER, is sufficient for the faithful reconstitution of estrogen signaling within these cells(18, 37). When expressed in yeast, the human ER will activate transcription from EREs located in reporter gene promoters in response to estradiol. This ability of ER to function within yeast allows a wide variety of genetic approaches to be taken toward defining the mechanisms of signal transduction and transcriptional regulation by the receptor.

To identify proteins that affect ER function, we have carried out a dosage suppression screen in yeast. In this technique, a mutant ER protein, with a reduced ability to activate transcription, is used as a substrate to isolate yeast gene product(s) that are capable of overcoming this mutant phenotype by favoring the interaction between ER and these factors, thus reconstituting receptor transcriptional activity. The mutant ER used in our dosage suppression screen contains a glycine to valine substitution at position 400 (G400V ER). This mutation is believed to alter the conformation of the ligand binding domain, which results in decreased hormone binding by the receptor, with a corresponding reduction in G400V ER's ability to activate transcription in response to estradiol(48). This mutant was selected as the substrate for the screen because it affects an early step in the ER signaling pathway, namely steroid binding, and therefore has the potential to result in the isolation of factors important for either steroid binding or transcriptional activation. We

anticipate that characterization of these proteins will ultimately give rise to a more complete understanding of the ER signal transduction pathway.

Using G400V ER as our dosage suppression screen substrate, we have isolated a yeast gene that, when overexpressed, is capable of increasing both G400V and wild type (wt) ER's ability to activate transcription in response to estradiol. This gene product is the yeast homologue of the vertebrate p23 protein(27), a component of the Hsp90-based molecular chaperone complex associated with unliganded steroid receptors as part of the aporeceptor complex. We have examined the functional relationship between p23 and ER *in vivo* under a range of receptor, estradiol and p23 concentrations. Our findings suggest that p23 is an important regulator of the ER signaling pathway.

6. BODY

Yeast dosage suppression screen.

To compare wt ER and G400V ER function in yeast, we constructed two strains containing a galactose-inducible expression vector, encoding either wt ER or G400V ER, and a reporter plasmid containing an ERE located upstream of the β-galactosidase gene. The transcriptional activities of wt ER and G400V ER, as a function of hormone concentration, were measured. Compared to wt ER, G400V ER requires a 100-fold increase in 17-β-estradiol concentration before receptor transcriptional activation is observed in our yeast assay (Figure 1A). At saturating ligand concentrations, however, G400V ER is able to reach the same maximal activity as wt ER, suggesting that once the block to steroid binding is overcome, the receptor is able to act as efficiently as wt ER in entering the functional interactions downstream of estradiol binding, both protein-protein and protein-DNA, that are necessary for transcriptional activation.

The G400V ER phenotype is most apparent at a concentration of 1 nM 17-β-estradiol, where wt ER shows maximal transcriptional response, but G400V ER displays only minimal transcriptional activity (Figure 1A). Exploiting this phenotypic difference, we carried out a dosage suppression screen to identify yeast proteins that, when overexpressed, would increase the transcriptional activity of G400V ER, thereby suppressing the mutant phenotype. The yeast strain containing G400V ER and an estrogen-responsive β-galactosidase reporter gene was transformed with a high-copy yeast genomic library and assayed for G400V ER transcriptional activity on X-gal indicator plates containing 1 nM 17-β-estradiol. Under these conditions, yeast colonies expressing the wt ER are blue, while the G400V ER-expressing yeast appear white (Figure 1B). Blue colonies were considered to be potential suppressor candidates. Five candidate

suppressors of the G400V ER phenotype were isolated after screening ~6000 colonies, which we estimate to represent about half of the yeast genome.

Identification of the yeast gene YKL 117w as a suppressor of the G400V ER phenotype.

One high-copy suppressor plasmid, designated 4.3, was found to increase G400V ER transcriptional activity 10-fold, bringing G400V ER transcriptional activation to one third the wt ER level at 1 nM 17-β-estradiol (Figure 1B and C). The 5' and 3' ends of the insert of the library plasmid were sequenced and aligned with the yeast genome. In this manner, we were able to identify the suppressor DNA as an 8,147 base pair genomic fragment of chromosome XI(25), containing multiple open reading frames (ORFs) (Figure 1D). To identify the suppressing ORF, a series of deletions were constructed and analyzed for their ability to increase G400V ER transcriptional activity. As seen in Figure 1D, the suppression of the G400V ER phenotype correlates with the presence of ORF YKL 117w.

Possible role of YKL 117w in ER signaling.

A search of the Swissprot database revealed YKL 117w to be the yeast homologue of the human p23 protein (yhp23)(27), a component of the Hsp90-based molecular chaperone complex. During preparation of this manuscript, two separate reports(2, 16), characterizing yeast strains deleted of YKL117w, have called this gene SBA1, reflecting an increased susceptibility of steroid signaling to benzoquinone ansamycin antibiotics. As the YKL117w gene product's homology to the vertebrate p23 protein was of considerable importance in investigating its role in ER function, we have chosen to refer to it as yhp23 to maintain this emphasis.

Although p23's specific function is not known, *in vitro* studies suggest that it is crucial to the stability of the aporeceptor complex of unliganded steroid receptors. Removal of p23 greatly reduces the formation of stable aporeceptor complexes of glucocorticoid(11, 12, 23) and progesterone receptors(28, 29) (GR and PR). In addition, *in vitro* studies have suggested that p23 possesses abilities typical of molecular chaperones, as it is capable of interacting with denatured β-galactosidase, suppressing its aggregation, and maintaining it in an intermediate, folding-competent conformation(17). The relative importance of these two aspects of p23 function in ER signaling has not been determined.

Characterization of yhp23's role in ER signaling.

Having identified yhp23 as the suppressing ORF, we cloned the YKL 117w gene, with an amino-terminal hemagglutinin (HA) epitope tag (HA-yhp23), into a yeast expression vector containing a constitutively active glycerol-phosphate dehydrogenase (GPD) promoter. We then established a yeast strain that overexpresses HA-yhp23 in the

presence of G400V ER, along with a reporter plasmid containing the β-galactosidase gene under control of an ERE. As seen in Figure 2A, overexpression of HA-yhp23 increases G400V ER transcriptional activity by 10-fold. Extracts from these yeast strains were prepared and analyzed by immunoblotting, using antibodies specific for ER and HA. Figure 2B demonstrates that overexpression of yhp23 does not affect the level of G400V ER protein and, therefore, the increase in transcriptional activity is not a result of increased receptor expression. The HA-tagged yhp23 protein migrates on an SDS-polyacrylamide gel at approximately 34 kDa. The higher molecular weight of the yeast protein (34kDa *vs* the 23kDa human protein) is expected, as the yhp23 is larger (216 residues) than its human counterpart (160 residues).

Given p23's chaperone-like activities *in vitro*, it could be argued that yhp23's interaction with the mutant G400V ER might arise as a function of the receptor's misfolded steroid binding domain, rather than reflect a true role in the ER signaling pathway. To determine whether yhp23 overexpression would have an effect on wild type steroid receptor activity, we constructed additional yeast strains that overexpress yhp23 in the presence of either wt ER or wt GR, along with reporter plasmids containing the β-galactosidase gene under control of the appropriate hormone response element, ERE or GRE, respectively. The effect of yhp23 overexpression upon ER and GR activity was then assayed using steroid concentrations where transcriptional induction by the receptors is approximately half of maximal levels: 0.1 nM 17-β-estradiol and 100 nM deoxycorticosterone (DOC), respectively. As seen in Figures 3C and D, overexpression of yhp23 increases the activity of both wt ER and wt GR, resulting in a greater than 50% increase in transcriptional activation in either case. The ability of yhp23 to functionally interact with wt ER and GR, and not just the mutant G400V ER, strongly implicates yhp23 as a member of the normal signaling pathway of ER and GR.

yhp23 increases ligand binding by G400V ER.

In light of p23's proposed role in aporeceptor complex formation, and given the nature of the G400V ER mutation, we examined whether suppression of the G400V ER phenotype results from increased estradiol binding by the receptor in the presence of overexpressed yhp23. Estradiol binding by G400V ER in the presence and absence of yhp23 overexpression was measured *in vivo*, using the yeast strains described in Figure 2A. Both yeast strains were incubated for one hour in media containing [^{3}H]-17-β-estradiol, washed three times to remove unbound steroid, and the amount of estradiol bound to G400V ER was measured by quantifying [^{3}H]-17-β-estradiol content by liquid scintillation counting.

Elevated yhp23 levels resulted in an increase in the total amount of steroid bound by G400V ER (Figure 3A and B). At a concentration of 1 μM 17-β-estradiol, G400V ER bound over three times more ligand in the presence of overexpressed yhp23. This effect was more pronounced at the lower concentration of 100 nM 17-β-estradiol, where yhp23 overexpression increased ligand binding over five-fold. Western blots of the corresponding yeast extracts indicate that increased steroid binding does not result from increased ER levels (Figure 3C). Thus, the contribution of yhp23 to G400V ER estradiol binding is inversely proportional to the concentration of hormone, suggesting that p23's importance to ER signaling would be greater at lower hormone concentrations.

The magnitude of yhp23's effect on ER signaling is a function of ER and estradiol concentrations.

Having demonstrated that overexpression of yhp23 increases ER transcriptional activity, we next asked if a decrease in yhp23 concentration would reduce ER activity. To this end, we compared wt ER transcriptional activity in the yhp23 knockout strain (KO) to that in the parental strain (PA)(2), as well as the parental strain ectopically expressing HA-yhp23 (PA+HA-yhp23 strain). In this way, we were able to assay ER function in 1) the absence of yhp23 (KO), 2) the presence of endogenous levels of yhp23 (PA), and 3) the presence of both endogenous yhp23 and overexpressed HA-yhp23 (PA+HA-yhp23). Both a wt ER expression vector and an ERE-β-galactosidase reporter plasmid were introduced into these three yeast strains and ER transcriptional activity was measured at 0.1 nM 17-β-estradiol in galactose/raffinose-containing media, conditions that induce high levels of ER protein expression. As shown in Figure 4A, in the absence of yhp23 (KO), ER signaling still occurs, demonstrating that ER is capable of functioning in a yhp23-independent manner. Endogenous levels of yhp23 (PA) results in a 50% increase in ER activity relative to the KO strain. The PA+HA-yhp23 strain, containing the highest yhp23 levels (Figure 4B), exhibits an even higher level of ER induction (2.7-fold) than that seen in the KO strain. This increase in ER activity is not a function of increased receptor levels, since ER levels were unchanged in the absence or presence of yhp23 (data not shown). Thus, ER transcriptional activity increases in direct proportion to the concentration of yhp23.

Having shown that ER activity increases as a function of yhp23 concentration, we next asked if the effect of yhp23 upon ER signaling is also a function of ER concentration. This was accomplished by repeating the above experiment in media containing raffinose, which results in low levels of ER expression. When ER transcriptional activity was assayed under these conditions, a similar pattern of yhp23-dependent ER activation was

observed: ER functions in the KO strain, and this activity increases as a function of yhp23 concentration (Figure 4C). However, the magnitude of the effect of yhp23 on ER transcriptional activation is greater at the lower ER concentration, such that overexpression of the yhp23 in the parental strain (PA+HA-yhp23) results in an almost five-fold increase in ER transcriptional activity relative to the KO strain, thereby doubling the induction seen at high ER concentrations (Figure 4D). Thus, the magnitude of yhp23's effect on ER transcriptional activity is greater at low, rather than high, ER concentration.

Our *in vivo* ligand binding assays suggest that the effect of yhp23 on estradiol binding by G400V ER is inversely proportional to hormone concentration. To further test this idea, we assayed the activity of wt ER within the KO and PA+HA-yhp23 strains at 0.1 nM and 1 nM 17-β-estradiol, under conditions of low ER expression. As seen in Figure 4E, a five-fold and two-fold induction in ER transcriptional activity is observed in the PA+yhp23 overexpression strain at 0.1 nM and 1 nM 17-β-estradiol, respectively. This trend continues when activities are compared at yet higher hormone concentrations, such that no difference in activity is observed between the KO and PA+HA-yhp23 strains at 10 nM 17-β-estradiol (not shown). Thus, the effect of yhp23 overexpression on ER transcriptional activation is inversely proportional to hormone concentration.

Interestingly, comparison of ER activity in the KO and PA+HA-yhp23 strains in the absence of estradiol suggests that overexpression of yhp23 also increases ER ligand-independent activity. As shown in Figure 4F, ER exhibits greater estradiol-independent activity in the presence of yhp23 overexpression, than in its absence. This effect on transcription is not observed in the absence of ER expression, suggesting that yhp23 is operating via ER to induce estradiol-independent transcriptional activation. This finding suggests that both estradiol-dependent and independent ER transcriptional activation are a function of yhp23 expression.

Thus, the relationship of yhp23 to ER signal transduction is dependent not only on concentrations of yhp23, but upon the level of ER and estradiol as well. Our data demonstrate that the effect of yhp23 upon ER function is most pronounced when ER activity is examined at low estradiol and ER concentrations.

yhp23 is not essential for the formation of a functional ER-GRIP1 complex.

The ER activates transcription in mammalian cells through two transcriptional activation domains, termed AF-1 and AF-2(35). The AF-1 domain, located within the amino terminus of the receptor, does not require steroid binding to achieve an active conformation(36). In contrast, the AF-2 domain lies within the steroid binding domain and is dependent upon estradiol binding for its activity(49). The ER AF-2 region has been shown to activate transcription in yeast and in cultured mammalian cells through interaction

with coactivator proteins including GRIP1/TIF2(20) and SRC1(39). These coactivators have no yeast homologues, and thus the AF-2 transcriptional activation domain of ER is largely inactive in yeast(21). Therefore, the possible role of yhp23 in AF-2 function has yet to be addressed.

The recent determination of the structure of the ER ligand binding domain bound to estradiol has suggested that proper folding of this region around the steroid hormone is crucial to the formation of an AF-2 domain competent for interaction with coactivators(3). As p23 has been described as having chaperone-like activities(17), it may play a role in the proper folding of the AF-2 domain around estradiol during the process of steroid binding. To determine whether yhp23 facilities ER AF-2-coactivator interactions, we introduced the mammalian coactivator GRIP1 into yhp23 KO and PA yeast strains expressing wt ER (KO+GRIP1 and PA+GRIP1, respectively). Coexpression of GRIP1 should activate AF-2, thereby increasing ER transcriptional activation relative to the control strains lacking GRIP1(21), and allowing us to compare GRIP1 induction of ER activity in the presence and absence of yhp23. If yhp23 were important for AF-2 interaction with GRIP1, then GRIP1-dependent ER transcriptional activation would be reduced in the KO *vs* the PA strain. However, as shown in Figure 5, this is not the case. ER transcriptional activation in the PA and KO strains is enhanced to similar extents (approximately 10-fold) over the corresponding control strains lacking GRIP1 (Figure 5). Thus, GRIP1-induction of ER transcriptional activation is not altered by yhp23 expression, suggesting that yhp23 is not required for the formation of a functional ER-GRIP1 complex.

ER and yhp23 colocalize within the nucleus of yeast.

Prior genetic and biochemical studies have demonstrated that aporeceptor complex formation is conserved in yeast(5). Hsp82, the yeast homologue of Hsp90, has been shown to associate with hormone-free ER and GR in yeast. In addition, genetic studies indicate that ER and GR signaling is reduced in yeast strains expressing only 5% of the wt level of Hsp82(40). Compelling genetic evidence also exists for the role of the yeast Hsp70(30), p60(8), Hsp40(30), and immunophilin(14) homologues in steroid signaling in yeast, with the majority of these proteins having been shown to associate with GR in the absence of hormone(2, 7).

Given human p23's presence in the aporeceptor complexes of PR(27) and GR(11), and having shown that yhp23 affects ER function, we proceeded to determine if yhp23 and ER colocalize *in vivo*. To determine the cellular distribution of yhp23 in yeast, we created a yhp23-green fluorescent protein (GFP) fusion protein by subcloning GFP at the carboxy-terminus of the yhp23 protein. Expression of the fusion protein was confirmed by Western

blot. Importantly, the yhp23-GFP fusion protein is also able to suppress the G400V ER phenotype (not shown), proving that addition of the GFP moiety does not eliminate yhp23's ability to functionally interact with G400V ER.

We constructed several yeast strains which express yhp23-GFP either alone, or in combination with G400V ER, wt ER, or wt GR. Figure 6A demonstrates that the distribution of yhp23-GFP in the absence of steroid receptor expression is largely cytoplasmic, though a small proportion of signal corresponding to the nucleus is also evident. This pattern is consistent with the expression pattern described for the human p23 protein within mammalian cells(27). Strikingly, upon coexpression of G400V ER, yhp23-GFP becomes predominantly limited to the nucleus, thus colocalizing with G400V ER (Figure 6B). Recall, that ER is a steroid receptor that resides in the nucleus in the absence of hormone. Importantly, this pattern of nuclear localization was not seen when G400V ER was coexpressed with just the GFP protein, indicating that yhp23 is responsible for the localization of the fusion protein to the nucleus (Figure 6C). Nuclear localization of yhp23-GFP was also observed when it was coexpressed with wt ER (Figure 6D). Thus, the ability of G400V ER to colocalize yhp23-GFP in the same manner as wt ER suggests that the G400V ER phenotype is not a result of deficient aporeceptor complex formation. Additionally, when cells coexpressing wt ER and yhp23-GFP were incubated in 17-β-estradiol, yhp23-GFP redistributed to the cytoplasm, reestablishing the pattern seen in yeast lacking ER expression (Figure 6A). As a final control, yhp23-GFP coexpressed with GR, a steroid receptor that exists outside the nucleus in the steroid-free state, did not localize to the nucleus (Figure 6F), but instead showed a cytoplasmic distribution similar to that of GR.

The colocalization of yhp23 and ER is consistent with the proposed role of yhp23 as a member of ER aporeceptor complex. Upon coexpression with ER, aporeceptor complex formation causes yhp23, presumably through an interaction with Hsp82 (the yeast homologue of Hsp90), to become localized to the nucleus. Addition of hormone appears to result in the dissociation of the aporeceptor complex, allowing yhp23 to redistribute throughout the cell.

Complementation of yhp23 by human p23 in yeast.

We next examined whether human p23, when ectopically expressed in yeast, functions like yhp23 to suppress the G400V ER phenotype. We established yeast strains that express G400V ER and an ERE-responsive reporter plasmid in the presence of HA-tagged human p23 (HA-p23) or HA-yhp23. A third strain containing the expression vector without an insert (vector) was used as a negative control. As shown in Figure 7A, human

p23 is capable of increasing hormone-dependent G400V ER transcriptional activation in yeast, though to a lesser degree than yhp23 (four-fold and 13-fold, respectively). The reduced G400V ER transcriptional activity is not a function of reduced human p23 expression relative to yhp23, since immunoblot analysis using an antibody directed against the HA-epitope present on both proteins show equal expression levels in yeast (Figure 7A, bottom panel). In addition, G400V ER expression is unaffected by yeast or human p23 coexpression (not shown). These findings suggest that human p23 can function in yeast similarly to yhp23, albeit less potently, to increase G400V ER transcriptional activity.

We next evaluated whether human p23 could function in yeast to increase ER transcriptional activation in the absence of endogenous yhp23. The KO strain expressing wt ER and an ERE-responsive promoter were transformed with 1) the empty expression vector; 2) HA-p23; or 3) HA-yhp23. When assayed at a concentration of 0.1 nM 17-β-estradiol, expression of human p23 increases ER transcriptional activity compared to vector only (Figure 7B), but to a lesser extent than yhp23 (two-fold and five-fold, respectively). Again, the reduced activity of ER in the human p23-expressing strain is not a function of reduced p23 levels relative to yhp23 (Figure 7B, bottom panel). These findings indicate that human p23 can partially complement the loss of yhp23 function in yeast with respect to ER signaling, thus suggesting that yhp23 and p23 are functional homologues.

Increased ER transcriptional activation by human p23 overexpression in MCF-7 cells.

To establish whether p23 affects ER signal transduction in mammalian cells, we examined the ability of human p23 to increase ER-mediated transcriptional enhancement when overexpressed in cultured mammalian cells. ER-containing MCF-7 cells were transfected with the reporter plasmid ERE-thymidine kinase-Luciferase and a plasmid encoding human p23. Transfected cells were treated with 0.1 nM 17-β-estradiol or ethanol vehicle for 24 hours, and transcriptional activity was quantified by luciferase activity. As shown in Figure 8, both hormone-dependent and hormone-independent ER transcriptional activity are increased roughly two-fold when p23 is overexpressed. This likely represents an underestimate of p23's importance to ER function, as these results are obtained in a cell line that contains endogenous p23 (not shown). These findings suggest that p23 is a limiting factor for ER signal transduction and therefore, subsequent ER-mediated transcriptional enhancement.

7. CONCLUSIONS

Using dosage suppression analysis in yeast to isolate factors involved in ER signal transduction, we have identified the yeast homologue of the human p23 (yhp23) as a

protein that, when overexpressed, results in a 10-fold increase in G400V ER transcriptional activation. *In vivo* estradiol binding assays suggest that yhp23 overexpression increases G400V ER transcriptional activity by increasing the number of estradiol-bound receptors. The effect of yhp23 overexpression was not limited to G400V ER, as it also increases the transcriptional activity of both wt ER and wt GR. This establishes yhp23 as a member of reconstituted steroid receptor signaling pathways in yeast.

Analysis of ER signaling as a function of yhp23, ER and estradiol concentrations demonstrates that the magnitude of the effect of yhp23 on ER transcriptional activation is inversely proportional to the concentration of both ER and estradiol. Thus, at low, sub-saturating concentrations of estradiol, yhp23 overexpression markedly increases ER transcriptional activation. In contrast, at saturating concentrations of estradiol, the effect of yhp23 overexpression on ER transcriptional activation is comparatively small. Furthermore, the magnitude of the effect of yhp23 on ER transcriptional activation is greater at low, rather than high, ER expression levels. Taken together, our findings indicate that the effect of yhp23's on ER signaling varies depending on yhp23, ER and estradiol concentrations.

Subcellular localization studies using a yhp23-GFP fusion protein indicates that yhp23 is largely cytoplasmic in the absence of ER expression. When coexpressed with ER, yhp23 colocalizes with the receptor to the nucleus in the absence of estradiol. This colocalization is reversed upon estradiol treatment, such that yhp23 is released into the cytoplasm. From these observations, we conclude that yhp23 is part of the ER aporeceptor complex in yeast and that the distribution of yhp23 within the cell is dynamic and affected both by ER expression and estradiol binding.

It has been proposed that the function of p23 in steroid receptor signaling is to promote, through its interaction with Hsp90, the maturation or stabilization of the aporeceptor complex(23, 29). This model, derived largely from *in vitro* experiments(41, 42), proposes that the heat shock proteins, Hsp90, p60, Hsp70, and possibly Hsp40(10), form a complex termed a "foldosome"(22), within which Hsp90 exists in a conformation incompatible with p23 binding (47)(Figure 9). The foldosome binds to the free receptor, which exists in a conformation with low affinity for ligand. In a process that requires ATP and monovalent cations, the Hsp90 component of the foldosome and the receptor undergo a conformational change(13), such that Hsp90 is now capable of binding p23(47), and the receptor exhibits high affinity steroid binding. p23 binding to Hsp90 appears to stabilize this immature aporeceptor complex *in vitro*. In the absence of p23, the Hsp90-receptor complex is inherently unstable and rapidly dissociates(13).

The ability of yhp23 to increase ER transcriptional activation might be expected if yhp23 is limiting for the formation of mature aporeceptor complexes. Increasing the concentration of yhp23 would result in a greater number of mature ER aporeceptor complexes in the cell. As a result, the total estradiol binding will increase, consistent with our *in vivo* estradiol binding assays (Figure 3). Thus, overexpression of yhp23, by increasing the number of mature aporeceptor complexes, will manifest itself as an increase in transcriptional activation by ER at a given hormone concentration (Figures 2 and 4). Thus, our *in vivo* findings are consistent with the current model of p23 function as derived from *in vitro* experiments.

Our results indicating that ER activity is detectable in the absence of yhp23, however, suggests that in addition to the p23-dependent pathway, there must also exist a p23-independent pathway leading to estradiol binding and signaling by ER (Figure 4). We propose that p23-independent activation of ER *in vivo* occurs through estradiol binding directly to the immature (p23-deficient) aporeceptor complex (Figure 9). This hypothesis is consistent with *in vitro* observations that in the absence of p23, the foldosome proteins Hsp90, Hsp70, and p60, are sufficient to induce the hormone-binding conformation of steroid receptors(13). We therefore propose that estradiol binding by ER is a composite of both the p23-independent and p23-dependent pathways. The relative contributions of each pathway to ER activation is dependent upon the concentration of p23, ER and estradiol (see below).

One prediction of our model is that yhp23 becomes less relevant to ER activation as the ratio of immature to mature aporeceptor complexes increases. The ratio of the two types of aporeceptor complexes is, in turn, a reflection of both ER and yhp23 concentration. Increasing ER expression when p23 levels are constant results in a greater number of the immature aporeceptor complexes, which favors hormone binding through the p23-independent pathway. Conversely, increasing yhp23 levels facilitates the formation of mature aporeceptor complexes, and therefore the p23-dependent pathway. This model of ER-signal transduction is consistent with our *in vivo* findings that indicate the magnitude of the effect of yhp23 on ER transcriptional activation is inversely proportional to the concentration of ER (Figure 4D).

The proposed model further envisions that the concentration of estradiol also affects the relative contribution of the p23-dependent and p23-independent steroid binding pathways. As suggested by Figure 9, free steroid can be considered to be competing with yhp23 for binding to the immature aporeceptor complex. As a result, the p23-independent pathway becomes more prominent as estradiol concentrations rise or, conversely, as yhp23 levels fall. Consistent with this notion, our data demonstrate that the magnitude of yhp23's

effect on ER signaling is greatest at low subsaturating, rather than high saturating, estradiol concentrations (Figure 4E). Thus, our model predicts that the balance among yhp23, ER, and estradiol, ultimately determines the relative contributions of the p23-dependent and p23-independent pathways to ER signal transduction.

Our model also provides insight into the observation that yhp23 overexpression induces G400V ER activity to a greater extent than that observed for wt ER (Figure 2). We suggest that by altering the steroid binding domain conformation, the G400V mutation largely eliminates the p23-independent pathway. G400V ER is temperature-sensitive, relative to the wt ER, for estradiol binding *in vitro*, displaying reduced estradiol binding at 25°C, but not at 4°C(48). This suggests that the G400V ER mutation destabilizes the conformation of the steroid binding domain, such that the receptor is unable to bind steroid with high affinity at 25°C. This mutation does not inhibit G400V ER's interaction with the aporeceptor complex, since 1) G400V ER has been reported to be complexed with Hsp90(1), and 2) our subcellular localization studies suggest G400V ER associates with yhp23 as efficiently as does wt ER. We therefore propose that the G400V mutation, by altering the structure of the steroid binding domain, largely inhibits estradiol binding to the transient, immature aporeceptor complex, thereby diminishing estradiol binding through the p23-independent pathway. As a result, yhp23 competes more effectively with estradiol for binding to the complex, favoring the p23-dependent estradiol binding pathway. The stability gained through yhp23 binding to the Hsp90-chaperone machinery, in turn, facilitates steroid binding by G400V ER.

Elegant genetic studies of yhp23 function in yeast by Bohen(2) and Fang *et al.* (16) demonstrate that yhp23 associates with Hsp90, and is a part of the GR aporeceptor complex in yeast. In contrast to our findings with ER, analysis of androgen receptor (AR) signaling in yeast suggests that it is largely p23-independent. This may reflect inherent differences in the mechanism of signal transduction employed by the receptors. Alternatively, we would suggest that, although the analysis of AR signaling was performed under a range of steroid concentrations, the levels of AR expression used may have favored the p23-independent pathway. It would be interesting to re-evaluate AR signaling as a function of yhp23 at both low AR and testosterone concentrations, conditions that would favor the p23-dependent pathway.

The partial complementation of human p23 in yeast lacking yhp23 strongly suggests that yhp23 functions as the p23 homologue with respect to ER signaling (Figure 7). Although the yeast and human p23 proteins share regions of identity, significant sequence differences between the proteins also exist(2). We speculate that this reflects species-specific differences in p23-Hsp90 association, and might therefore explain the

inability of human p23 to fully complement the loss of yhp23 function. It would be interesting to examine whether yeast expressing human Hsp90(40) and p23 increase ER activity to the same extent as their yeast counterparts.

Finally, our studies also provide insight into the possible mechanisms by which the ER communicates with other signaling pathways. Unexpectedly, a significant increase in estradiol-independent activation of ER was observed as a result of yhp23 overexpression (Figure 4F). This estradiol-independent activation of ER was also observed upon p23 overexpression in MCF-7 cells (Figure 8). Previous studies have proposed that estradiol-independent transcriptional activation by ER results, in part, from ER phosphorylation through an EGF-dependent pathway(4, 26). Thus, the most direct interpretation of our data suggests that maintenance of ER within the aporeceptor complex facilitates (but is not essential to) estradiol-independent activity, perhaps by maintaining ER in a conformation amenable to phosphorylation.

In addition to ER, several other signaling molecules (including c-Src and c-Raf) are dependent upon chaperone complexes for their function(41). Yeast deficient in the DnaJ homologue YDJ1, for instance, display both altered steroid receptor and Src kinase activity(30). Thus, molecular chaperones link diverse signaling pathways. Additional insight into the mechanism of this cross-talk comes from our subcellular localization studies that reveal a striking colocalization of ER and yhp23 within the nucleus. Estradiol treatment was shown to liberate yhp23 (and presumably other chaperone proteins) from the nucleus, allowing it to redistribute throughout the cytoplasm, where it can potentially interact with other signaling proteins. Although our colocalization studies were carried out under conditions of overexpression, we speculate that estradiol activation of ER may, through the release of chaperone components, modulate the activity of a variety chaperone-dependent pathways. In light of p23's role in stabilizing chaperone complexes, it is likely to play a key regulatory role in any such "chaperone signaling".

In conclusion, we have provided evidence that yhp23 is a member of the ER signaling pathway and a positive regulator of ER function. We also suggest that at high ER and/or estradiol concentrations, conditions often present in the yeast system or during transient overexpression of ER in cultured mammalian cells, ER signaling occurs largely through a p23-independent pathway. Under low physiological concentrations of ER and estradiol, p23 is likely to be an important regulator of ER. Our results also indicate that alterations in the level or subcellular distribution of p23 are potential mechanisms for modulating estradiol-dependent and independent ER transcriptional activation. It remains to be examined whether p23 levels or subcellular distribution fluctuate between normal and tumor cells, during cellular proliferation or differentiation or upon growth factor treatment.

Conceivably, the concentration of p23 or its subcellular localization may be an important contributing factor in estrogen-related malignancies, and therefore a potential point of intervention and treatment. As aporeceptor complex formation is also believed to be important for ligand binding by GR, PR, and AR, p23 will likely play an important role in these pathways as well. Thus, the ability to control steroid receptor signaling through the manipulation of p23 function may have utility for the treatment of multiple medical conditions.

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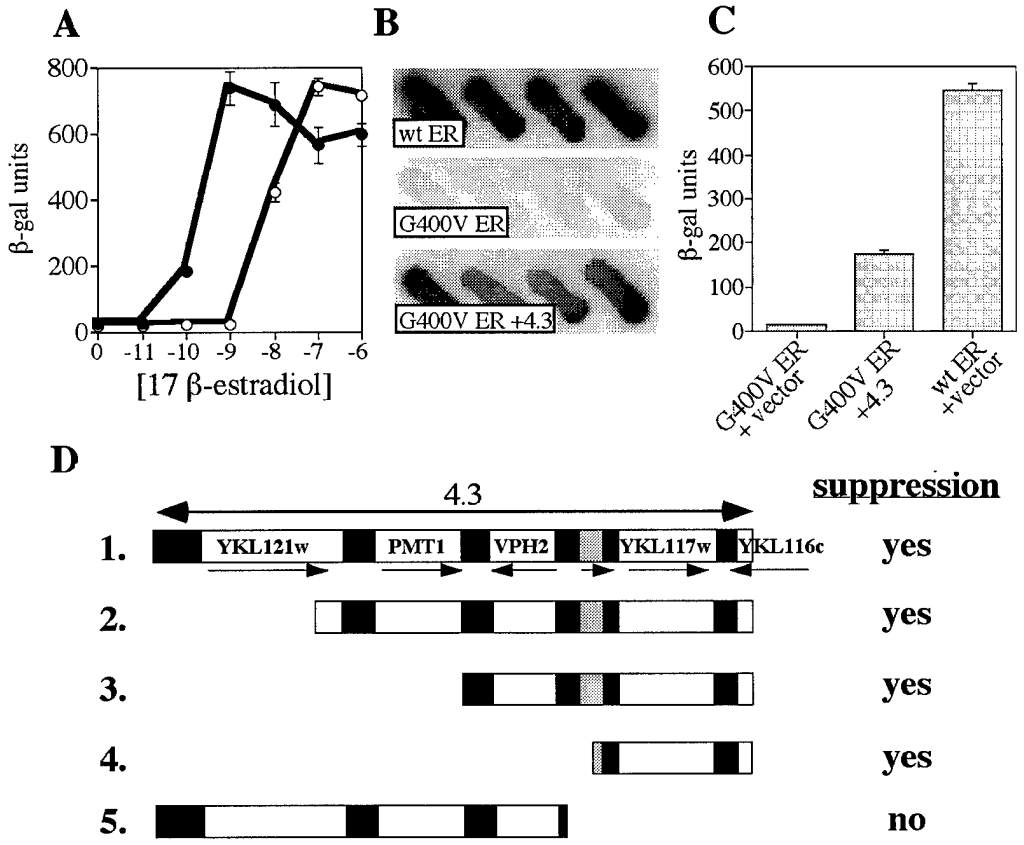
9. APPENDICES (Figures and Figure Legends)

Figure 1: Isolation of a yeast genomic fragment that suppresses the G400V ER phenotype.

A) Transcriptional activity of wt ER and G400V ER as a function of 17-β-estradiol concentration. The W303a yeast strain was transformed with a galactose-inducible expression vector containing either wt or G400V ER, along with an ERE-containing β-galactosidase reporter plasmid. Transcriptional activation by wt ER (filled circles) and G400V ER (open circles) in response to increasing 17-β-estradiol concentration was determined by liquid β-galactosidase assay as described in Materials and Methods. Note that G400V ER requires a hundred-fold higher estradiol concentration to induce transcriptional activation compared to wt ER. The dosage suppression screen was carried out in the presence of 1 nM 17-β-estradiol, the conditions under which the G400V ER phenotype is most pronounced. B) The relative activity of wt ER, G400V ER, and G400V ER + suppressor 4.3. Four independent colonies on X-Gal indicator plates containing 1

nM 17-β-estradiol are shown and represent wt ER + empty library plasmid (wt ER); G400V ER + empty library plasmid (G400V ER); and G400V ER + suppressor plasmid 4.3 (G400V ER + 4.3). C) Transcriptional activity of wt ER, G400V ER, and G400V ER in presence of suppressor 4.3. Liquid β-gal assays were performed on yeast strains containing wt ER + empty library plasmid, G400V ER + empty library plasmid, and G400V ER + 4.3, at 1 nM 17-β-estradiol. Suppressor 4.3 increases G400V ER activity ten-fold, bringing its activity to nearly one third that of wt ER. D) Identification of YKL 117w as the G400V ER suppressor. The sequence of the yeast genomic fragment contained within the suppresser 4.3 was determined by aligning the 5' and 3' ends of the insert to the yeast genomic sequence database. Suppressor 4.3 contains a 8,147 bp fragment comprising 4 complete open reading frames (YKL 121w, PMT1, VPH2, and YKL 117w), a partial open reading frame (YKL 116w), and a tRNA-Ala gene (shaded box). The relative positions and orientation of the genes within the 4.3 fragment are shown schematically (fragment 1). Identification of the gene responsible for suppressing the G400V ER phenotype was accomplished by constructing 5' and 3' deletion derivatives of the 4.3 suppressor (fragments 2-5) and assaying their ability to increase G400V ER transcriptional activity at 1 nM 17 β-estradiol. "Yes" indicates the fragment is capable of suppressing the G400V ER phenotype, while "No" represents fragments that fail to increase G400V ER transcriptional activity. YKL 117w was present within the suppressing fragments (1-4), but absent within the fragment that did not suppress (5), suggesting that its gene product is responsible for increasing G400V ER transcriptional activation. YKL 117w encodes the yeast homologue of the human p23 protein (yhp23/SBA1).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G400V ER +4.3
600
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300
200
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β-gal units
G400V ER + vector
G400V ER +4.3
wt ER + vector
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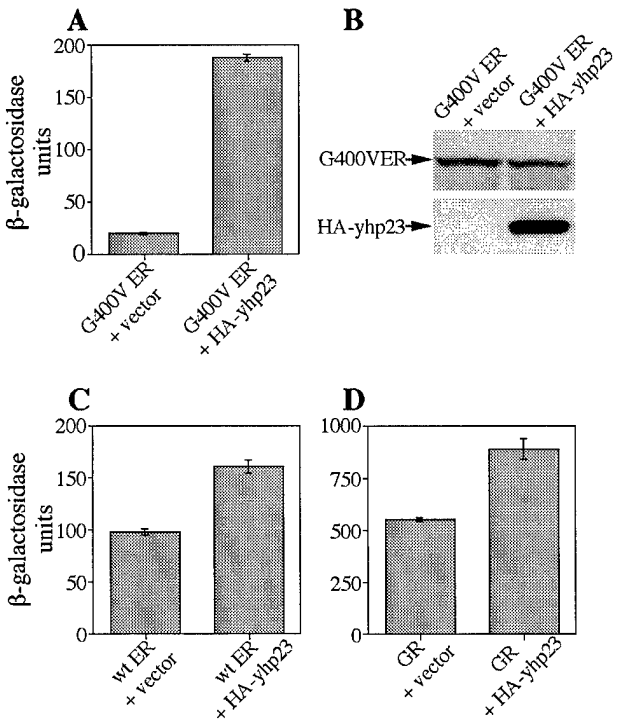
4.3
suppression
1.
YKL121w
PMT1
VPH2
YKL117w
YKL116c
yes
2.
yes
3.
yes
4.
yes
5.
no

Figure 2: Effect of yhp23 overexpression on G400V ER, wt ER, and GR transcriptional activity.

An HA-tagged yhp23 gene was cloned into the yeast expression vector pRS314 downstream of the GPD promoter. Yeast strains were then constructed which coexpress G400V ER, wt ER, or GR and the HA-tagged yhp23, in the presence of a β-galactosidase reporter gene under the control of the appropriate hormone response element (ERE or GRE). A) Overexpression of HA-yhp23 increases G400V ER transcriptional activation. The activity of G400V ER in the presence and absence of overexpressed yhp23 was determined by liquid β-galactosidase assay at a concentration of 1 nM 17-β-estradiol. Overexpression of yhp23 increased G400V ER activity approximately ten-fold. B) Increased transcriptional activation of G400V ER by yhp23 is not a function of increased ER levels. Whole cell lysates were prepared from the yeast strains described in (A). Equal amounts of proteins were separated on SDS/4-20% gradient polyacrylamide gel, transferred to Immobilon paper, and probed with an ER-specific rabbit polyclonal antiserum (top panel) or a monoclonal antibody directed against the HA-epitope on yhp23 (bottom panel) and visualized by enhanced chemiluminescence. C) The activity of wt ER in the absence and presence of yhp23 at a concentration of 0.1 nM 17-β-estradiol. D) The activity of GR in the absence and presence of yhp23 at a concentration of 100 nM DOC.

Fig. 2


A
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C
D
β-galactosidase units
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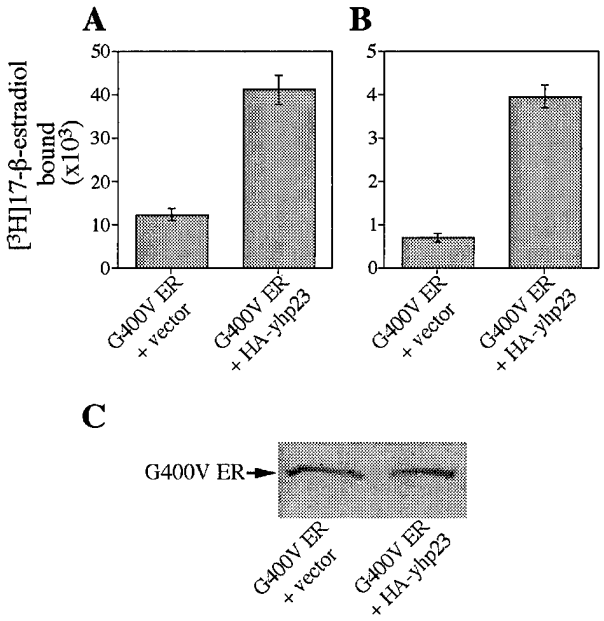
100
50
0
G400V ER + vector
G400V ER + HA-yhp23
G400VER
HA-yhp23
wt ER + vector
wt ER + HA-yhp23
1000
750
500
250
GR + vector
GR + HA-yhp23

Figure 3: Overexpression of yhp23 increases estradiol binding by G400V ER *in vivo.*

The total amount of estradiol bound by G400V ER in the presence and absence of yhp23 overexpression was determined by *in vivo* ligand binding assay, using the two yeast strains described in Figure 2A. After a one hour incubation in the presence of (A) 1 μM or (B) 100 nM [^{3}H]-labeled 17-β-estradiol, cells were washed to remove unbound ligand, and amount of bound estradiol was determined by liquid scintillation counting. C) ER expression in not altered by HA-yhp23. Whole cell extracts from yeast strains described in A, were fractionated on SDS-PAGE and ER expression was examined by immunoblotting with an ER-specific rabbit polyclonal antiserum as described in Materials and Methods.

Fig. 3


A
B
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[3H]17-β-estradiol bound (x10^3)
50
40
30
20
10
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5
4
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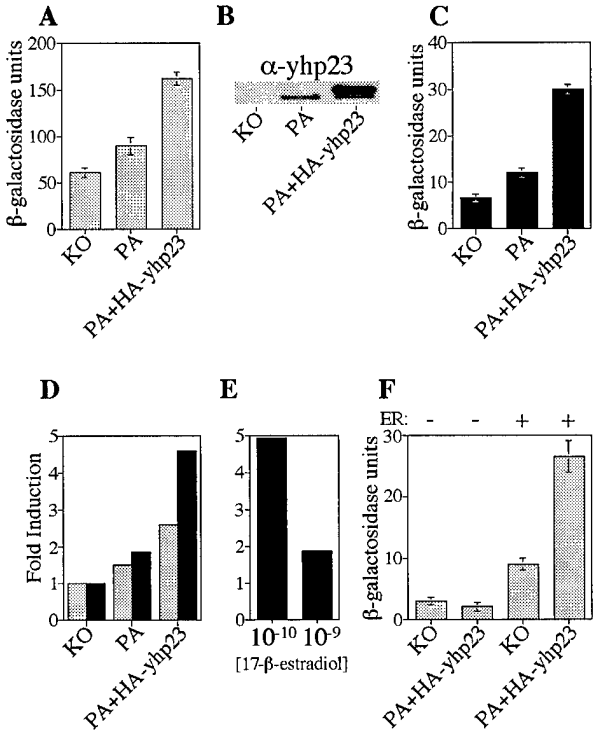
1
0
G400V ER + vector
G400V ER + HA-yhp23
G400V ER
G400V ER + vector
G400V ER + HA-yhp23
G400V ER + vector
G400V ER + HA-yhp23

Figure 4: Yhp23's effect on ER activity is a function of yhp23, ER, and estradiol concentrations.

ER transcriptional activation was compared in three yeast strains expressing yhp23 at different concentrations. The knockout strain (KO) is yhp23 deficient, the parental strain (PA) expresses yhp23 at endogenous levels, and the HA-yhp23-transformed parental strain (PA+HA-yhp23) expresses endogenous yhp23 and exogenous HA-yhp23. All four strains express wt ER under the control of a galactose-inducible promoter, along with an ERE-β-galactosidase reporter plasmid. A) The effect of yhp23 on ER transcriptional activation at high ER concentrations. ER transcriptional activity as a function of yhp23 concentration was determined by liquid β-galactosidase assay in cells incubated in galactose/raffinose-supplemented media containing 0.1 nM 17-β-estradiol. B) KO, PA and PA+HA-yhp23 strains express different levels of yhp23. Equal amounts of whole cell lysates from the KO, PA, and PA+HA-yhp23 strains were analyzed by immunoblotting using anti-yhp23 polyclonal antibody as described in Materials and Methods. The upper band seen in the PA+HA-yhp23 lane corresponds to the HA-tagged yhp23. C) The effect of yhp23 on ER transcriptional activity at low ER concentration. ER transcriptional activity as a function of yhp23 concentration was determined by liquid β-galactosidase assay in cells incubated in raffinose-supplemented media containing 0.1 nM 17-β-estradiol. Under these conditions ER expression is 10-times lower as compared to cells grown in the presence of galactose (not shown). D) Yhp23 induction of ER transcriptional activity is inversely proportional to ER concentration. The fold induction of yhp23 on ER transcriptional activity at 0.1 nM 17-β-estradiol under conditions of high ER expression (galactose, gray bars) or low ER expression (raffinose, black bars) is standardized to the ER activity in the KO strain. E) The effect of yhp23 on ER function is inversely proportional to ligand concentration. The fold induction of ER transcriptional activation in the PA+HA-yhp23 *vs* KO strains at 0.1 nM and 1 nM 17-β-estradiol was determined under conditions of low ER expression (raffinose media). Results indicate that the fold-induction of ER transcriptional activation in the presence of yhp23 overexpression is significantly greater at 0.1 nM 17-β-estradiol than 1 nM 17-β-estradiol. F) Yhp23 overexpression increases estradiol-independent ER transcriptional activation. The effect of yhp23 overexpression on ERE-dependent transcriptional activation in the presence and absence of ER is shown. KO and PA+HA-yhp23 strains containing the ERE-reporter construct, were transformed with either the empty expression vector or with the ER-containing expression vector and assayed for β-galactosidase activity in galactose/raffinose media without estradiol.

Fig. 4


A
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β-galactosidase units
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PA+HA-yhp23
α-yhp23
40
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D
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5
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1
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ER: - - + +

Figure 5: Yhp23 is not essential for functional interaction between ER and the coactivator GRIP1.

Yhp23-KO and PA strains were constructed that express wt ER both in the presence and absence of the coactivator GRIP1. ER activity was assayed in the four strains after incubation in raffinose media containing 0.1 nM 17-β-estradiol. Data were collected in the same experiment but are displayed with separate y-axis to more clearly demonstrate GRIP1 induction of ER activity within each strain. Coexpression of GRIP1 increases ER transcriptional activation in both KO and PA strains approximately 10-fold, an effect that is independent of yhp23 expression. Note that yhp23 increased ER transcriptional activation to the same extent in the absence or presence of GRIP1.

Fig. 5

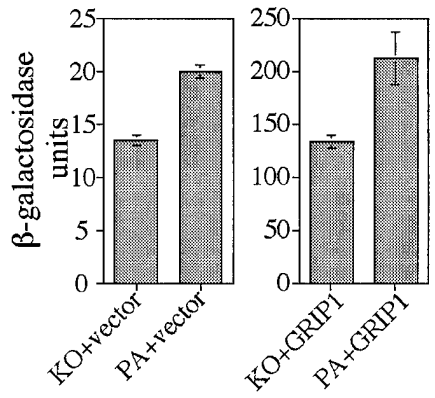

β-galactosidase units
25
20
15
10
5
0
250
200
150
100
50
0
KO+vector
PA+vector
KO+GRIP1
PA+GRIP1

Figure 6: ER and yhp23-GFP colocalize within the nucleus of yeast.

To examine yhp23 subcellular localization in yeast, a yhp23-GFP fusion protein was constructed. Yeast strains were created that express yhp23-GFP (A) or GFP (C) either alone, or in combination with G400V ER (B-C), wt ER (D-E), or GR (F). Cells were grown in galactose/raffinose-containing media, in the absence or presence of 17-β-estradiol. Cells were fixed, permeabilized, and incubated with the appropriate receptor primary antibody, a corresponding Texas-red-conjugated secondary antibody and the DNA in the nucleus was stained with Hoechst dye H334211. The GFP, Texas-red and Hoechst fluorescent signals were visualized using a Zeiss Axioplan 2 Fluorescent microscope. Note that yhp23-GFP is expressed throughout the cytoplasm in the absence of ER expression. In the presence of coexpressed G400V ER as well as wt ER, yhp23-GFP becomes localized to the nucleus. Incubation of the wt ER strain in 1 μM 17-β-estradiol results in the redistribution of yhp23-GFP from the nucleus to the cytoplasm, thereby reversing the ER-yhp23-GFP colocalization observed in the absence of estradiol.

Fig. 6

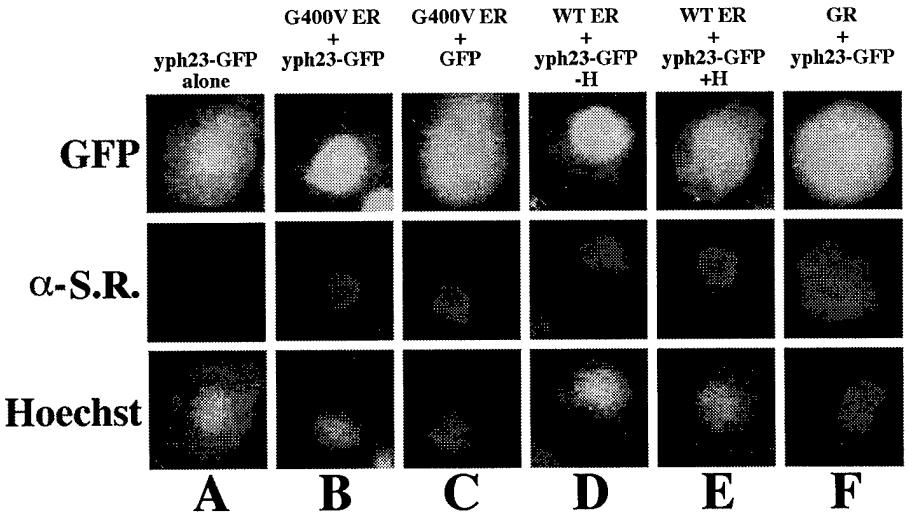

yph23-GFP alone
G400V ER + yph23-GFP
G400V ER + GFP
WT ER + yph23-GFP -H
WT ER + yph23-GFP +H
GR + yph23-GFP
GFP
α-S.R.
Hoechst
A
B
C
D
E
F

Figure 7: Complementation of yhp23 by human p23 in yeast.

A) Overexpression of HA-p23 suppresses the G400V ER phenotype. An HA-epitope tagged human p23 (HA-p23) was subcloned into the yeast expression vector pRS316-GPD. The W303a yeast strain expressing G400V ER and an ERE-β-galactosidase reporter gene was transformed with expression vectors containing 1) no insert (vector), 2) HA-p23, or 3) HA-yhp23. G400V ER transcriptional activity was measured by liquid β-galactosidase assay in galactose-containing media with 1 nM 17-β-estradiol. Equal amounts of whole cell lysates from the strain containing vector, HA-p23 and HA-yhp23 were analyzed by immunoblotting using anti-HA antibody as described in Materials and Methods (bottom panel). B) Human p23 partially complements the loss of yhp23 with respect to ER signaling. Yhp23-KO yeast strain expressing wt ER and an ERE-responsive β-galactosidase reporter gene were transformed with expression vectors containing 1) no insert (vector), 2) HA-p23, or 3) HA-yhp23. ER transcriptional activity was determined by liquid β-galactosidase assay in raffinose media containing 0.1 nM 17-β-estradiol. Immunoblot analysis for HA-p23 and HA-yhp23 was performed as in A and demonstrates that HA-p23 and HA-yhp23 are expressed at similar levels (bottom panel).

Fig. 7

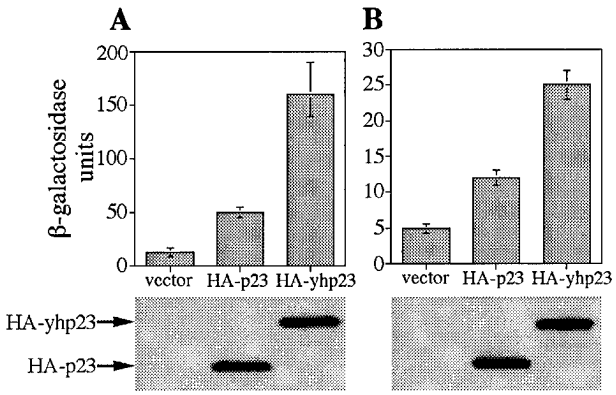

A
B
β-galactosidase units
200
150
100
50
0
30
25
20
15
10
5
0
vector
HA-p23
HA-yhp23
vector
HA-p23
HA-yhp23
HA-yhp23
HA-p23

Figure 8: Activation of ER transcriptional enhancement by ectopic p23 expression.

ER-containing MCF-7 cells (2.5 x 10^5 cells/6 cm dish) were transiently transfected using the lipid Trans-IT 100 with 5 μg of the ERE-containing luciferase reporter plasmid and 2 μg of a pCMV expression vector (white) or pCMV-HA-p23 expression vector (grey). Cells were incubated for 24 hours in the presence of 0.01 nM 17-β-estradiol or ethanol vehicle and harvested. ER transcriptional activation was measured by luciferase assay, normalized to total protein concentration in each sample and expressed as relative luminescence units (RLU). Data represents the mean of an experiment done in triplicate, which was repeated four times.

Fig. 8

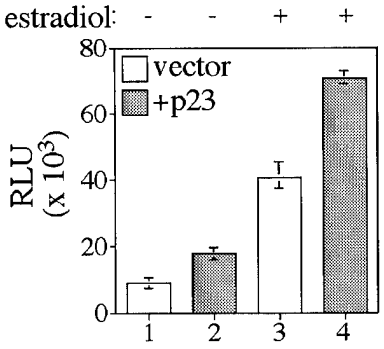
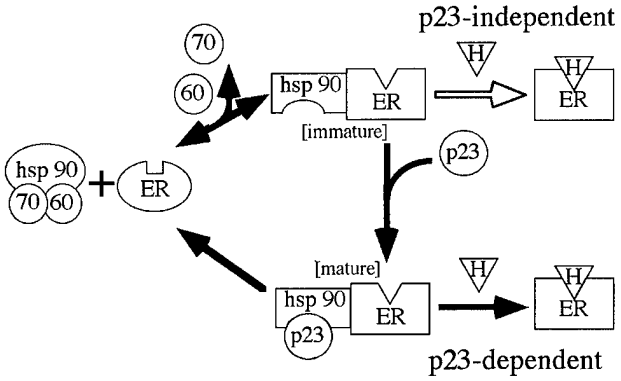


Figure 9: A model for p23-dependent and independent ER signal transduction.

A simplified version of the current model of aporeceptor complex formation as deduced from *in vitro* studies is indicated by the black arrows. "Hsp90" refers to the heat shock protein 90 dimer, "70" refers to heat shock protein 70, "60" refers to p60 , "p23" refers to the mammalian p23 protein, "ER" refers to the estrogen receptor, and "H" refers to hormone. The immunophilins, which do not appear essential to aporeceptor complex formation, but are isolated with the complex *in vivo*, have been excluded from the model for simplicity. According to the model, p60, Hsp70 (and possibly Hsp40), and a dimer of Hsp90 preassociate to form the "foldosome" complex. The foldosome binds to the steroid binding domain of the naive receptor. Hsp70 and p60 are released from the complex in a process that requires ATP and a monovalent cation, while Hsp90 and the receptor undergo conformational changes, such that the receptor assumes a conformation with high affinity for steroid. This new complex (labeled "immature" in figure), is inherently unstable and quickly disassociates unless p23 binds to Hsp90, thereby stabilizing the aporeceptor complex (labeled "mature" in figure). Our data, however, suggests that a second, p23-independent pathway to ER ligand binding exists *in vivo*, occurring when ligand binds directly to the immature aporeceptor complex (light gray arrow), which is favored at high ER and/or estradiol concentrations. We suggest that the G400V ER mutation renders the receptor less capable of participating in the p23-independent pathway, thereby functionally uncoupling the p23-independent and dependent pathways.

Fig. 9


p23-independent
70
60
hsp 90
ER
H
H
ER
[immature]
p23
hsp 90
70
60
ER
[mature]
H
H
hsp 90
ER
ER
p23
p23-dependent